Heterogeneity in *P. graminea* and *P. teres* f. *maculate* as revealed by IRAP analysis

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ABSTRACT

Barley-pathogenic *Pyrenophora* isolates are *P. graminea* (Pg) and *P. teres* f. *maculata* (Ptm), which cause foliar leaf stripe and net blotch lesions, respectively. However, the species are often indistinguishable by morphological and cultural characteristics. In this study, inter-retrotransposon amplified polymorphism (IRAP) analysis has been used to study the genetic relationship amongst 14 Pg and 14 Ptm isolates from distant geographical locations. A total of 592 scorable DNA bands were obtained 236 of these (39.43%) were polymorphic. A neighbour-joining diagram, based on Nei's genetic distances, separated the isolates into three main clusters, corresponding to the two pathogens studied except one isolate of each placed mixed. No clear geographical sub-structuring was found. The results confirmed that IRAP is a reliable, efficient, and effective marker technology for determining genetic relationships in *Pyrenophora* spp.

Keywords: Barley leaf stripe, net blotch, inter-retrotransposon amplified polymorphism, genetic diversity.

INTRODUCTION

*Pyrenophora graminea* Ito and Kurib. [anamorph *Drechslera graminea* (Raben. ex Sclecht.) Shoem.] and *Pyrenophora teres* Drechs. [anamorph *Drechslera teres* f. *maculata* (Sacc.) Shoem.] are serious seed-borne pathogens of barley, causing foliar leaf stripe and net blotch lesions, respectively (Mathre, 1997). However, the species are often indistinguishable by morphological and cultural characteristics (Smedegaard-Petersen, 1978; Mathre, 1997).

Pathogenicity markers are often limited in number and subject to host selection (Leung et al., 1993). Molecular approaches have been therefore developed for fungal systematic studies, arbitrary primers (Reeves and Ball, 1991), random amplified polymorphic DNA (RAPD) analysis (Jawhar et al., 2000), DNA sequencing of the ribosomal DNA spacer regions (Stevens et al. 1998) and of glycerolde-3-phosphate dehydrogenase gene (Zhang and Berbee, 2001). However, the methods more currently used are often based on the analysis of retrotransposons whose integration into new sites can be used as genetic markers (Taylor et al., 2004; Pasquali et al., 2007).

The retrotransposons markers have been shown to be a suitable method for taxonomic studies in *Alternaria alternata* (Kaneko et al., 2000), *Phytophthora parasitica* (Liou et al., 2002) and other fungi (Daboussi, 1996). Retrotransposons markers can be exploited by the IRAP (Inter-Retrotransposon Amplified Polymorphism) technique that amplifies bands from two nearby LTRs (long...
terminal repeats) using outward-facing primers annealing to LTR target sequences (Kalendar et al., 1999).

The objectives of the current research were (i) to investigate variability within the *Pyrenophora* spp. with respect to the retrotransposons elements using IRAP marker, and (ii) to determine whether or not the observed variability can be used to clarify inter-specific relationships among *Pyrenophora* isolates.

**MATERIALS AND METHODS**

**Fungal isolates**

Fourteen Syrian *P. graminea* (Pg) isolates described by Arabi and Jawhar (2007), and 10 Algerian (received from the university of sciences, Algeria) and 4 Syrian isolates of *P. teres f. maculata* (Ptm) were used in the study (Table 1). Leaf tissues with necrotic lesions were cut into small pieces (5 mm long) and surface sterilized in 5% sodium hypochlorite (NaOCl) for 5 min. After three washings with sterile distilled water, the pieces were transferred onto Petri dishes containing potato dextrose agar (PDA, DIFCO, Detroit, MI. USA) amended with 13 mg/l kanamycin sulphate, and incubated for 10 days, at 21 ±1ºC with a photoperiod of 12 h using near UV light for seven days or until sporulation occurred. Twenty–eight monosporic *Pyrenophora* isolates used in this study, it was possible to distinguish *P. graminea* from *P. teres f. maculata* by observing colony morphology and shape of conidia from microscopic analysis. Colony colour was recorded using an empirical scale from 1 (white) to 5 (black) (Table 1).

**DNA Extraction**

Fungal isolates were cultured for 2 weeks at 20ºC on PDA and stored at 4 ºC for further study. Mycelium was harvested and DNA was extracted according to standard protocols (Leach et al., 1986), resuspended in TE buffer (10 mM Tris–HCl, pH 8.0; 1mM EDTA) and stored at -20ºC.

**Inter-retrotransposon polymorphism (IRAP) analysis**

The IRAP method was carried out as previously described by using LTR primers derived from the barley genome (Kalendar et al., 1999). The primer sequences, retrotransposon type, and orientation are shown in Table 2. The reactions of 20 µl contained: 0.075 M Tris-HCl pH 8.8, 0.02 M (NH₄)₂SO₄, 1.5 mM MgCl₂, 0.01% Tween-20, 0.2 mM dNTPs, 0.2 µM each primer, 1.5 U Taq polymerase, and 10 ng DNA template. PCR was performed in a Thermocycler (BIO–RAD system, USA). The amplification program consisted in an initial denaturation step at 95 ºC for 4 min, followed by 35 cycles of denaturation for 1 min at 95ºC, annealing for 1 min (temperatures are specified in Table 3) and extension for 2 min. at 72 ºC. A final extension of 72 ºC for 5 min was incorporated into the program, followed by cooling to 4 ºC until recovery of the samples. PCR products were visualized using UV light and separated on a 2% agarose gel following staining with ethidium bromide. The sizes of the amplified products were estimated by comparison with the molecular weight of the DNA ladder marker (Q.BIOgene).

**Statistical analysis**

Each amplified fragment was treated as a unit character and scored as a binary code 1 and 0 for presence and absence, respectively, using 1Dscan EX 3.1software (Scanalytics, Inc.). The experiments were repeated twice for each isolate to confirm the repeatability and the monomorphic bands were removed from the analysis. The data were analyzed using Treecon software (Van de Peer and De Wachter, 1994). A phylogenetic tree was
Table 1. *Fusarium* isolates, host, location, colony colour and IRAP polymorphism.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Host</th>
<th>Location</th>
<th>Year of collection</th>
<th>Colony colour</th>
<th>Total number of bands</th>
<th>Polymorphic Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. gramineae (Pg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>barley leaves</td>
<td>ICARDA (north)</td>
<td>1996</td>
<td>5</td>
<td>21</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>barley leaves</td>
<td>Syria (south)</td>
<td>2002</td>
<td>3</td>
<td>23</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>barley leaves</td>
<td>Syria (south)</td>
<td>1997</td>
<td>4</td>
<td>23</td>
<td>13</td>
</tr>
<tr>
<td>4</td>
<td>barley leaves</td>
<td>Syria (north-east)</td>
<td>2002</td>
<td>4</td>
<td>21</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>barley seeds</td>
<td>Syria (south)</td>
<td>2002</td>
<td>3</td>
<td>25</td>
<td>8</td>
</tr>
<tr>
<td>6</td>
<td>barley leaves</td>
<td>Syria (north-east)</td>
<td>2006</td>
<td>5</td>
<td>24</td>
<td>11</td>
</tr>
<tr>
<td>7</td>
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<td>Syria (north-east)</td>
<td>1997</td>
<td>3</td>
<td>20</td>
<td>7</td>
</tr>
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<td>2003</td>
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<td>Syria (south)</td>
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<td>3</td>
<td>20</td>
<td>8</td>
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<tr>
<td>11</td>
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<td>Syria (south)</td>
<td>1997</td>
<td>3</td>
<td>18</td>
<td>7</td>
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<tr>
<td>12</td>
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<td>7</td>
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<tr>
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<td>3</td>
<td>17</td>
<td>6</td>
</tr>
<tr>
<td>14</td>
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<td>2003</td>
<td>2</td>
<td>21</td>
<td>11</td>
</tr>
<tr>
<td>P. teres f. maculata (Ftm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1</td>
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<td>Algeria</td>
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<td>2005</td>
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<td>20</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
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<td>Algeria</td>
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<td>3</td>
<td>23</td>
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<tr>
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<td>Algeria</td>
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<td>2</td>
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<tr>
<td>6</td>
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<td>Algeria</td>
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<tr>
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<td>2004</td>
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<td>Algeria</td>
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<tr>
<td>10</td>
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<td>Algeria</td>
<td>2006</td>
<td>4</td>
<td>17</td>
<td>7</td>
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<tr>
<td>11</td>
<td>barley leaves</td>
<td>Syria (north)</td>
<td>2001</td>
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<tr>
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<td>barley leaves</td>
<td>Syria (north-east)</td>
<td>2004</td>
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<td>23</td>
<td>7</td>
</tr>
<tr>
<td>13</td>
<td>barley leaves</td>
<td>Syria (east)</td>
<td>1997</td>
<td>3</td>
<td>20</td>
<td>6</td>
</tr>
<tr>
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<td>barley leaves</td>
<td>ICARDA (north)</td>
<td>2004</td>
<td>3</td>
<td>20</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td>521</td>
<td>236</td>
<td>39.43%</td>
</tr>
</tbody>
</table>

*ICARDA, International Center for Agricultural Research in Dry Areas, Syria.

1: white, 2: white grey, 3: grey, 4: grey black and 5: block.

Table 2. Primer name, retrotransposon type, position and sequence.

<table>
<thead>
<tr>
<th>Name and orientation type</th>
<th>Retrotransposon type</th>
<th>Accession</th>
<th>Position</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>3LTR (1)</td>
<td>EABE-I</td>
<td>217332-217332</td>
<td>2112-2138</td>
<td>TGGTTCCTCAGTGAGCCAAAAAATTATT</td>
</tr>
<tr>
<td>LTR6146 (2)</td>
<td>EABE-I</td>
<td>217332-217332</td>
<td>1993-2012</td>
<td>CGGTTCCTCAGTGAGCCAAAAAATTATT</td>
</tr>
<tr>
<td>LTR7210 (3)</td>
<td>EABE-I</td>
<td>217332</td>
<td>418-439</td>
<td>CTGGTTCCTCAGTGAGCCAAAAAATTATT</td>
</tr>
<tr>
<td>3LTR (1)</td>
<td>EABE-I</td>
<td>217332</td>
<td>1-52</td>
<td>TGGTTCCTCAGTGAGCCAAAAAATTATT</td>
</tr>
<tr>
<td>3LTR (1)</td>
<td>EABE-I</td>
<td>217332</td>
<td>314-338</td>
<td>CGGTTCCTCAGTGAGCCAAAAAATTATT</td>
</tr>
<tr>
<td>Sukkula</td>
<td>Sukkula</td>
<td>AY054373</td>
<td>4501-4526</td>
<td>GTAGAGGTTCCTCAGTGAGCCAAAAAATTATT</td>
</tr>
<tr>
<td>Nubata</td>
<td>Nubata</td>
<td>AY078074</td>
<td>1-22</td>
<td>COCATTCCTCAGTGAGCCAAAAAATTATT</td>
</tr>
<tr>
<td>Nubata</td>
<td>Nubata</td>
<td>AY078075</td>
<td>1-22</td>
<td>COCATTCCTCAGTGAGCCAAAAAATTATT</td>
</tr>
</tbody>
</table>
constructed using the neighbour-joining algorithm (Saitou and Nei, 987) by the PHYLIP package ver 3.5c (Felsenstein, 1993).

RESULTS AND DISCUSSION

Seven out of 28 possible combinations successfully amplified fragments from the genomic DNA of all studied P. genome isolates. These primer combinations produced many distinctively amplified fragments ranging in size from 100 to 1800bp. Table 1 shows that a total of 592 bands were scored, of which 236 (39.43 %) were polymorphic.

Distance analysis of IRAP data clearly divided isolates of Pyrenophora species into three main groups, corresponding to the two pathogens studied, except one isolate of each (Pg1 and Ptm13) placed differently (Fig.1). However, the isolates displayed close genetic

Table 3. Annealing temperatures (°C) of primer combinations

<table>
<thead>
<tr>
<th>3'LTR</th>
<th>LTR6140</th>
<th>LRT6150</th>
<th>5'LRT1</th>
<th>5'LRT2</th>
<th>Sukkula</th>
<th>Nikita</th>
</tr>
</thead>
<tbody>
<tr>
<td>3'LTR</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>LTR6140</td>
<td>0</td>
<td>0</td>
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<td>LRT6150</td>
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<tr>
<td>5'LRT1</td>
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<td>0</td>
<td>0</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5'LRT2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sukkula</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nikita</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

0 unsuccessful primer combinations

Fig. 1. Neighbour-joining tree inferred from IRAP markers in 14 isolates of P. graminea (Pg) and 14 of P. teres f. maculata (Ptm).
relationship, i.e., over very long geographic
distances. Six of Algerian \textit{P. teres} f. \textit{maculate} isolates were closely grouped to
three Syrian isolates (Fig.1). A genetic study
on five populations of \textit{P. teres} from the USA
and Germany (Peever and Milgroom, 1994)
suggested that they were derived from a
common source population even though there
was a high level of genetic variation within
each population from random sexual
reproduction. However, the low interspecific
IRAP variation between \textit{P. graminea} and \textit{P. teres} f. \textit{maculata}, also suggests that these
species may have coevolved from a common
source pathogen.

Our results demonstrated that the two
species (isolates Pt\textit{m}13 and Pg1) were placed
in the same cluster (Fig 1). This might be
discussed to the fact that the genetic
relationships among \textit{Pyrenophora} species
could be attributed either to a higher
evolutionary rate in these species (“fast
evolutionary clock”) or to an earlier
branching in the Pyrenomycetes (Zhang and
Berbee, 2001). However, the studied species
played a prominent role in plant diseases of
diverse host; therefore a more comprehensive
study of each group is warranted. The present
results confirm the findings of Arabi et al.
(2006) and Arabi and Jawhar (2007) on the
genetic diversity of Syrian \textit{P. graminea}. Although different polypeptide and DNA
patterns were observed among the fungal
isolates, the authors suggested the use of
other markers to better clarify genetic
diversity in Syrian \textit{P. graminea} populations.
In this study, IRAP was applied and new
types of polymorphism were identified.

Different colony colours were observed
among isolates after 10 days of growth on
PDA media, but these were not correlated
with colony origin within the country (Table
1). Similar results have been previously
reported by Gatti et al. (1992) and Peever and
Milgroom (1994). Additionally, Zriba and
Harrabi (1995) attributed the morphological
changes to the effects of different
environmental conditions and considered
constant environmental conditions necessary
for taxonomic studies in \textit{Pyrenophora}. It is
thought that some characters gradually
disappeared with changes in the culture
conditions while others did not develop.

CONCLUSION
IRAP markers provide a rich source of
molecular markers which are rapid and
suitable way to group \textit{Pyrenophora} isolates
and to estimate the genetic relationships
between the groups, and it can be considered
a complement to the morphological and
mating studies for identification of
\textit{Pyrenophora} species. However, data from
the present work can support further studies
to uncover the effect of retrotransposons on
the structural and functional evolution of
genes in \textit{Pyrenophora} species.

ACKNOWLEDGEMENTS
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REFERENCES
Arabi, M.I.E., Jawhar, M. and MirAli, N.
2006. Polypeptide patterns of Syrian

Heterogeneity in \textit{Pyrenophora graminea}
as revealed by ITS-RFLP. \textit{J. Plant Pathol.}
89: 391-395.

Daboussi, M.J. 1996. Fungal transposable
elements: generators of diversity and

Gatti, A., Rizza, F., Delogu, G., Terzi, V.,
Physiological and biochemical variability

Felsentein, J. 1993. \textsc{Phylip} (Phylogeny
Inference Package) version 3.5c.
Department of Genetics, University of Washington, Seattle, WA, USA.


